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Mal-Development of the Penis and Loss of Fertility in Male Rats Treated Neonatally with Female Contraceptive 17α-Ethinyl Estradiol: A Dose-Response Study and a Comparative Study with a Known Estrogenic Teratogen Diethylstilbestrol

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The objectives of this study were to find a minimal dose of 17α-ethinyl estradiol (EE) that is detrimental to the developing penis and fertility and to compare estrogenic effects between EE and diethylstilbestrol (DES). Neonatal rats received EE at 10 ng (1 μg/kg), 100 ng, 1 μg, or 10 μg per pup on alternate days from postnatal days 1 to 11 (dose-response study) or received EE or DES at 100 ng per pup daily from postnatal days 1 to 6 (comparative study). Effects of EE were dose dependent, with ≥ 100-ng dose inducing significant ($p < 0.05$) reductions in penile length, weight, and diameter. Additionally, the penis was malformed, characterized by underdeveloped os penis and accumulation of fat cells. Fertility was 0% in the ≥ 1-μg groups, in contrast to 60% in the 100-ng group and 100% in the 10-ng and control groups. Animals treated with ≥ 10 ng had significant reductions in the weight of bulbospongious muscle, testis, seminal vesicle, epididymal fat pad, and in epididymal sperm numbers. A comparison of EE and DES effects showed similar reductions in penile weight and length and the weight of bulbospongiosus muscle, testis, seminal vesicle, epididymis, and epididymal fat pad in both adolescent and adult rats. While 5/6 control males sired, only 1/6 in the EE group and 0/6 in the DES group sired. Hence, neonatal exposure to EE at 10 ng (environmentally relevant dose) adversely affects male reproductive organs. A dose ten times higher than this leads to permanently mal-developed penis and infertility. Furthermore, EE and DES exposures show similar level of toxicity to male reproductive organs.

Key Words: **ethinyl estradiol; DES; penis; development; toxicology.**

Exposure to environmental estrogens has been linked with increasing frequency of reproductive disorders (Toppari *et al.*, 1996). More than 2 million male offspring of women exposed to diethylstilbestrol (DES) during pregnancy have higher incidence of testicular cancer, cryptorchidism, hypospadias, and smaller penis (Brouwers *et al.*, 2006; Gill *et al.*, 1979). Laboratory animals exposed perinatally to estrogens develop higher incidence of male reproductive tract abnormalities, including hypospadias (Newbold, 2004). Sons of Danish women exposed to endocrine disrupting pesticides have smaller testes and penises (Anderson *et al.*, 2008). Alligators from Lake Apopka (FL) contaminated with industrial estrogenic chemicals have smaller phallus (Guillette *et al.*, 1996). All these findings underscore the long-term adverse effects of environmental estrogens on human and wildlife health.

A number of studies have raised concerns regarding the human health effects of bisphenol A (BPA), an estrogenic compound with its worldwide use in plastics and epoxy resins. The perinatal exposure to BPA at environmentally relevant doses (≤ 50 μg/kg) permanently increased the size of the prostate gland in adult CD-1 mice (Gupta, 2000), increased the number of prostatic ducts and an overall volume of the prostate gland in 19 gestation day CD-1 mice fetuses (Timms *et al.*, 2005), increased androgen-binding activity (Gupta, 2000) and androgen receptor (AR) gene expression (Richter *et al.*, 2007) in mesenchymal cells of the prostatic region of the urogenital sinus, and predisposed the prostate gland to a precancerous growth at adulthood (Prins *et al.*, 2007). Conversely, similar studies in the case of 17α-ethinyl estradiol (EE), a synthetic estrogen with its worldwide use in contraceptive pills, are limited and controversial (Howdeshell *et al.*, 2008; Sawaki *et al.*, 2003; Thayer *et al.*, 2001). More than 50 million women worldwide take contraceptive pills and 3–4% of them inadvertently continue to take the pills during the first trimester of pregnancy (Smithells, 1981). In this connection, the epidemiological data showing higher incidence of reproductive abnormalities in the offspring of women exposed to DES in the early part of pregnancy than in the later part (Swan, 2000) raise the possibility for the early embryo being vulnerable to EE exposure.

Supporting the above possibility are experimental observations of induction of hypospadias in mice by maternal exposure

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to EE and DES from 12 to 17 days of gestation (Kim *et al.*, 2004). The hypospadias resulted from a deficit in the gross morphogenesis of the penis where urethral folds fail to fuse properly. Gross morphogenesis is complete by birth in the rat penis, which is composed of a body and a glans. The body is further composed of two corpora cavernosa and a corpus spongiosus. However, histologically, the body is still made of stromal cells, whose differentiation into cavernous spaces (also called sinusoids) and smooth muscle cells does not commence until 4–6 days of age (Murakami, 1987). In this regard, the following observations from our laboratory are noteworthy. Neonatal DES exposure prior to 12 days of age, but not later, resulted in malformation of the rat penis, characterized by accumulation of fat cells and loss of cavernous spaces and smooth muscle cells in the body (Goyal *et al.*, 2005a). Furthermore, the magnitude of these effects was more pronounced in rats treated from postnatal days 1 to 6 than from postnatal days 7 to 12. Hence, these findings suggest that the rat penis is very sensitive to estrogen exposure during postnatal days 1–6, the period just before the beginning of stromal cell differentiation into cavernous spaces and smooth muscle cells.

Information as to the timing of differentiation of cavernous spaces and smooth muscle cells in the body of the human penis is not available in the literature, but it is most likely to occur soon after the completion of gross morphogenesis of the penis at the end of the first trimester and the beginning of the second trimester of the pregnancy (Klonisch *et al.*, 2004). This roughly corresponds to the period of human development when more than 2 million women worldwide inadvertently continue to take contraceptive pills during pregnancy and corresponds in the rat to postnatal days 1–6 of penis development. Hence, the objectives of this study, using our neonatal DES rat model (Goyal *et al.*, 2005a,b), are twofold: (1) to determine a minimal dose of EE exposure to neonatal pups that is detrimental to the penis, other male reproductive organs, and fertility and (2) to compare estrogenic effects between EE and a known estrogenic teratogen DES.

MATERIALS AND METHODS

Animals and housing. Neonatal and/or adult Sprague-Dawley male and female rats (Harlan Sprague Dawley, Indianapolis, IN) were maintained at 22°C–23°C ambient temperature, 55–60% relative humidity, and 12:12 light-dark cycle, and had free access to food (Rodent Chow 5001; Purina Mills, St Louis, MO) and water for 24 h. Animals were handled in accordance with the guidelines of the National Institutes of Health Guiding Principles for the Care and Use of Animal Research, and all animal procedures were approved by the Institutional Animal Care and Use Committee at Tuskegee University. Timed-pregnant female rats were housed individually. Within 24 h of delivery, five to eight male pups from different litters (one pup from each litter, in order to avoid a litter effect) were assigned to each group, and the number of pups per group was adjusted to eight with extra female pups, where appropriate.

Dose-response study. The objective of this study was to find a minimal dose of EE (Sigma, St Louis, MO) that adversely affects the developing penis and fertility. Five to six male pups per group received 25 μl sc injections of olive oil containing EE at a dose of 10 ng (1 μg/kg), 100 ng, 1 μg, or 10 μg per pup, per day, on alternate days, from postnatal days 1 to 11 (postnatal day 0 being the day of birth). Controls received oil only. The maximal 10-μg dose was selected based on our previous study, where all male pups treated at this dose with DES or estradiol valerate were infertile, and the loss of fertility was associated with a permanently malformed penis (Goyal *et al.*, 2005b). The minimal 10-ng dose was used because it roughly corresponds to the clinical dose of EE (35–50 μg) present in contraceptive pills taken by young women weighing 40–50 kg. A dose lower than 10 ng was not included because neither DES nor estradiol valerate at a dose of 1 ng caused any adverse effects on any of the male reproductive parameters (Goyal *et al.*, 2005b). Animals were weaned at 22 days of age and examined for various parameters during development as described below. Tissues were collected following asphyxiation with CO_2 at 140–150 days of age. One of five animals in the 1-μg group died before weaning.

EE and DES comparative study. The objective of this study was to compare effects of EE with those of DES, which is a synthetic, nonsteroidal estrogen with known toxic effects in reproductive organs of both sexes. Five to eight male pups per group received daily 25 μl sc injections of olive oil containing EE or DES at a dose of 100 ng per pup, per day, from postnatal days 1 to 6. The above dose and duration of treatment were selected because this treatment regimen in our previous studies caused permanent penile maldevelopment and loss of fertility in at least 50% of the adult rats treated neonatally with DES (Goyal *et al.*, 2005a,b). Animals were weaned at 22 days of age and examined for various parameters during development as described below. Tissues were collected following asphyxiation with CO_2 at 48–50 days and 140–150 days of age. The reason for collecting tissues at two different time periods of growth was to compare effects of these compounds at adolescence and adult stages of the animal.

Descent of testes and release of preputial sheath. In the dose-response study, the testis descent was examined once per week from 22 to 57 days of age; and the preputial sheath was examined once per week from 43 to 71 days of age. In the comparative study, the testis descent was examined every other day from 22 to 30 days of age and the preputial sheath was examined every third day from 43 to 70 days of age. Testes were characterized as fully descended when they were palpated in the scrotum while holding the animal in a supine position. The formation of a scrotal bulge marked the descent. The preputial sheath was characterized as fully released when it completely retracted from the glans penis up to its transition with the shaft of the penis.

Fertility. Four to five adult male rats (110–120 days of age) from each of the 10 μg, 1 μg, 100 ng, 10 ng, and control groups in the dose-response study were transferred to mating cages floored with a mesh grid and cohabited with untreated, 60- to 70-day-old females (1:1) for 12 days. Cages were checked twice daily for the presence of copulatory plugs. The plug-positive females were separated and evaluated for the presence of sperm in vaginal washings. Females were allowed to deliver and the number of pups per litter was recorded. A similar fertility trial was run in adult rats of the comparative study.

Body and organ weights. All animals were weighed immediately after CO_2 asphyxiation. The testis, head and body of the epididymis, tail of the epididymis, and seminal vesicle (including coagulating gland) of both sides were weighed. While testes and epididymides were trimmed of fat prior to taking weights, seminal vesicles were weighed with their secretions intact in the alveoli. In addition, the epididymal fat pad, located between the tail of the epididymis and the distal extremity of the testis, was weighed. This parameter had been found to be as sensitive as the weight of seminal vesicles to neonatal estrogen exposure in our previous dose- (Goyal *et al.*, 2005b) and time-response (Goyal *et al.*, 2005a) studies.

The penis was measured for length, diameter, and weight. The prepuce was retracted, and the body of the penis was exposed up to the ischial arch, the point of origin of the root of the penis. The stretched length was measured from the tip of the glans penis to the midpoint of the ischial arch and the diameter from the middle of the body of the penis with a caliper (calibrations up to 0.1 mm). After removing the free loose connective tissue, the entire penis was weighed.

Additionally, to assess the status of the os penis development, one penis per group was radiographed as described previously from our laboratory (Goyal *et al*., 2005b). The penile skeletal muscles, including the bulbospongiosus and levator ani, were isolated and weighed.

Histopathology and histochemistry. After weighing, tissues (4–5 mm thick) from the testes were fixed in Bouin's fluid, and tissues from the epididymis and the middle part of the body of the penis were fixed in 10% formaldehyde for 24–48 h. Tissues were processed for paraffin embedding using an automatic tissue processor; 5-μm-thick paraffin sections were stained with hematoxylin and eosin (H&E) and examined for routine histopathology using a light microscope. In addition, for fat demonstration in the body of the penis, formaldehyde-fixed tissues were en block stained for 8 h with 1% osmium tetroxide dissolved in 2.5% potassium dichromate solution and then processed for paraffin embedding; 5-μm thick osmium-fixed, unstained, undeparaffinized sections of the body of the penis were examined using light microscopy, and the adjacent serial sections were stained with H&E to allow for examination of histopathology. Such examination of undeparaffinized and unstained sections is useful in viewing the full extent of fat infiltration in the penis because it rules out the possibility of fat droplets being washed out during H&E staining. Four to five animals from each group of the dose-response and comparative studies were used for histopathology and histochemistry examination.

Digital images of histopathological and histochemical sections, as well as of gross specimens of the penis, were captured using a Leitz Orthoplan microscope (Vashaw Scientific, Inc., Norcross, GA) and Kodak Microscopy Documentation System 290 (Eastman Kodak Company, Rochester, NY) and were assembled using Adobe Photoshop CS2.

Epididymal sperm numbers. Epididymal sperm numbers, a measure of efficiency of spermatogenesis, were counted in the dose-response study only. The left epididymis was collected at the time of necropsy and frozen at −20°C. After thawing, the head and body and the tail of the epididymis were each homogenized in 50 ml PBS, as described previously from our laboratory (Goyal *et al*., 2005a). The homogenate was filtered through a metal sieve, and a 200-μl aliquot of the filtrate was mixed with 100 μl of PBS and 200 μl of 1% trypan blue, which stains sperm heads. An 8.5-μl aliquot of the stained filtrate was used to count the number of sperm heads in each sample in duplicate using a hemocytometer.

Plasma testosterone. Plasma testosterone was measured in rats of the dose-response study only. One blood sample was collected from the heart of each animal prior to necropsy, and plasma was frozen at −20°C. Testosterone was measured using a COAT-A-COUNT testosterone radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) according to manufacturer's protocol. The sensitivity of the assay was 0.2 ng/ml. All samples were quantified in a single assay, and the intra-assay coefficient of variation was 7%.

Statistics. Statistical analyses were performed using ProStat statistical software (Polysoftware International, Pearl River, NY). Analysis of variance was performed on all parameters except those from the mating study. Treatment groups with means significantly different ($p < 0.05$) were identified using Duncan's multiple range test. When data were not distributed normally, or heterogeneity of variance was identified, analyses were performed on transformed or ranked data. Data from the mating study were evaluated using Fisher's exact test. Data are expressed as mean ± SE throughout the text.

RESULTS

Body Weight

In the dose-response study, the body weight at adulthood was similar between controls and all the treated groups, except in the 10-μg group where it was reduced by almost 20% (Fig. 1A, Table 1). In the comparative study, the body weight was also similar between controls and the treated animals, regardless whether they were treated with EE or DES or whether they were adolescent or adult (Fig. 3A, Table 2).

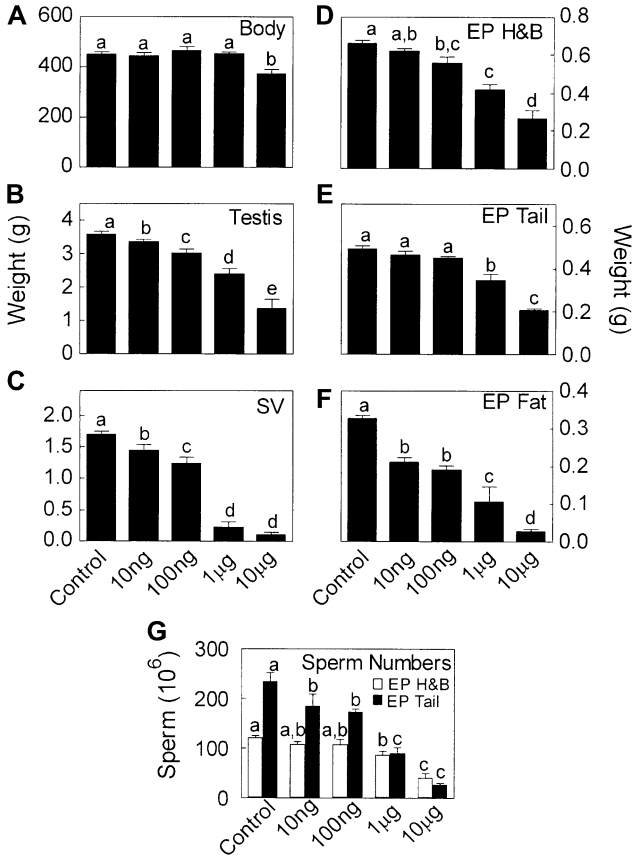

FIG. 1. The body weight (A), the absolute paired weight of the testis (B), seminal vesicle (C), head and body of the epididymis (D), tail of the epididymis (E), and epididymal fat pad (F), and the number of sperm in the head and body and the tail of the epididymis (G) in adult rats treated neonatally with EE at a dose of 10 ng (1 μg/kg) or 100 ng or 1 μg or 10 μg/rat on every other day from postnatal days 1 to 11. Note a significant reduction in the weight of the testis, seminal vesicle, and epididymal fat pad and sperm numbers in the tail of the epididymis in the > 10-ng groups. Data are expressed as mean ± SEM. Means with different superscripts are significantly ($p < 0.05$) different from controls. SV, seminal vesicle; EP, epididymis; H&B, head and body.

Organ Weight

Testis, epididymis, and seminal vesicle. In the dose-response study, the absolute weight of the testis, seminal vesicle, and head and body of the epididymis decreased significantly in a dose-dependent manner; however, comparatively, the rate of decrease was much higher for the seminal vesicle (Figs. 1A–D). For example, the decrease was almost 90% in the seminal vesicle, versus 60% in the testis, versus 50% in the head and body of the epididymis in the 10-μg group, compared with controls. The weight of the tail of the epididymis also decreased in all treated groups; but the decrease was significant only in the case of the 1-μg and

TABLE 1

Effects of Neonatal EE Exposure at Various Concentrations on the BW, the Paired Relative Weight (100 g BW) of the Testis, SV, HB/EP, T/EP, EP Fat, and the Relative Weight of the BS, LA, and Penis

Groups	BW (g)	Testis (g)	SV (g)	HB/EP (g)	T/EP (g)	EP Fat (g)	BS (g)	LA (g)	Penis (g)
Control	451 ± 10^{A}	0.7995 ± 0.0332^{A}	0.3780 ± 0.0193^{A}	0.1509 ± 0.0057^{A}	0.1099 ± 0.0047^{A}	0.0724 ± 0.0004^{A}	0.2273 ± 0.0083^{A}	0.0609 ± 0.0028^{A}	0.0644 ± 0.0014^{A}
10 ng	446 ± 11^{A}	0.7573 ± 0.0236^{A}	0.3225 ± 0.0201^{B}	0.1436 ± 0.0034^{A}	0.1038 ±0.0030^{B}	0.0472 ± 0.0020^{B}	0.1968 ± 0.0070^{B}	0.0618 ± 0.0048^{B}	0.0656 ± 0.0016^{B}
100 ng	467 ± 16^{A}	0.6508 ± 0.0296^{B}	0.2645 ± 0.0235^{C}	0.1230 ± 0.0076^{B}	0.0967 ± 0.0038^{B}	0.0409 ± 0.0018^{C}	0.1650 ± 0.0097^{C}	0.0578 ± 0.0037^{A}	0.0513 ± 0.0007^{B}
1 μg	454 ± 8^{A}	0.5256 ± 0.0227^{C}	0.0592 ± 0.0200^{D}	0.1074 ± 0.0045^{B}	0.0751 ± 0.0053^{C}	0.0235 ± 0.0089^{C}	0.0847 ± 0.0190^{D}	0.0462 ± 0.0036^{B}	0.0314 ± 0.0019^{C}
10 μg	375 ± 17^{B}	0.3704 ± 0.0551^{D}	0.0293 ± 0.0066^{D}	0.0766 ± 0.0109^{C}	0.0573 ± 0.0016^{D}	0.0072 ± 0.0013^{D}	0.0402 ± 0.0064^{E}	0.0346 ± 0.0037^{B}	0.0241 ± 0.0007^{D}

Note. BW, body weight; BS, bulbospongious; LA, levator ani; SV, seminal vesicle; HB/EP, head and body of the epididymis; T/EP, tail of the epididymis; EP fat, epididymal fat pad. Data are expressed as mean ± SEM. Means within columns with different superscripts (A–E) are significantly different ($p < 0.05$).

10-μg groups (Fig. 1E). The relative weight (per 100 g body weight) of all three organs also showed dose-dependent declines at levels more or less similar to those observed in the absolute weight (Table 1). The right or left testis from one animal each of the 1-μg and 10-μg groups was attached to the scrotal wall and could not be separated from the adjacent head and body of the epididymis. Likewise, the tail of the epididymis was not weighed in one animal of the 1-μg group and three animals of the 10-μg group because it had an abscess in one or both sides of these animals.

In the comparative study, both EE and DES treatments caused reductions in the absolute weight of the testis, seminal vesicle, and epididymis; however, the level of reduction differed among organs, as well as between the adolescent and adult stages of development. For example, compared to controls, the testis weight decreased by 7–15% in both treatments and developmental stages, but the decrease was significant only in the DES treatment (Fig. 3B). Conversely, while the weight of the seminal vesicle in both treatments decreased by 62–68% at adolescence, it decreased only by 27–34% at adulthood (Fig. 3C). The weight of the head and body of the epididymis decreased by 7–8% in both treatments and developmental stages, but the decrease was significant only at adulthood in the DES treatment (Fig. 3D). The weight of the tail of the epididymis did not significantly differ from controls, whether animals were treated with EE or DES or whether they were adolescent or adult (Fig. 3E). The relative weight of all three organs showed differences in levels of reductions among organs, as well as between treatments and stages of development, more or less similar to those observed in the absolute weight (Table 2). The weight of the testis, head and body of the epididymis, and tail of the epididymis was not recorded in one animal each of the DES and EE groups because the testis and head and body of the epididymis were encased in a thick capsule and were difficult to separate, and the tail of the epididymis of one or both sides had an abscess.

Epididymal fat pad. Both absolute and relative weights of the epididymal fat pad, located between the tail of the epididymis and the distal extremity of the testis, decreased significantly in a dose-dependent manner (Fig. 1F, Table 1), decreasing from 60 to 64% of the control level in the minimal dose group (10 ng) to 8–10% of the control level in the maximal dose group (10 μg). The weight was not recorded in one animal of the 1-μg group and three animals of the 10-μg group because of the difficulty to separate the fat pad from the adjoining tail of the epididymis, which was abscessed on one or

TABLE 2

A Comparison of Effects of Neonatal EE and DES Exposure on the BW, the Paired Relative Weight (100 g BW) of the Testis, SV, HB/EP, T/EP, and EP Fat, and the Relative Weight of the BS, LA, and Penis in Adolescent and Adult Rats

Group	BW (g)	Testis (g)	SV (g)	HB/EP (g)	T/EP (g)	EP Fat (g)	BS (g)	LA (g)	Penis (g)
Adolescent									
Control	217 ± 11	1.1475 ±0.0251^{a}	0.1376 ± 0.0151^{a}	0.0975 ± 0.0039	0.0479 ± 0.0021^{a}	0.0227 ± 0.0007^{a}	0.1351 ± 0.0038^{a}	0.0421 ± 0.0018	0.0740 ± 0.0035^{a}
DES, 100 ng	228 ± 8	0.9279 ± 0.0331^{c}	0.0519 ± 0.0038^{b}	0.0851 ±0.0033	0.0363 ± 0.0011^{c}	0.0141 ± 0.0009^{b}	0.0804 ± 0.0056^{b}	0.0376 ± 0.0080	0.0453 ± 0.0032^{b}
EE, 100 ng	222 ± 4	1.0248 ± 0.0215^{b}	0.0637 ± 0.0054^{b}	0.0877 ± 0.0042	0.0430 ± 0.0013^{b}	0.0118 ± 0.0010^{b}	0.0819 ± 0.0062^{b}	0.0381 ± 0.0029	0.0469 ± 0.0027^{b}
Adult									
Control	456 ± 16	0.8789 ± 0.0241^{A}	0.3304 ± 0.0101^{A}	0.1579 ± 0.0031^{A}	0.1041 ± 0.0014^{A}	0.0700 ± 0.0050^{A}	0.1856 ± 0.0048^{A}	0.0551 ± 0.0013	0.0569 ± 0.0018^{A}
DES, 100 ng	481 ± 9	0.7428 ± 0.0197^{B}	0.2234 ± 0.0176^{B}	0.1376 ± 0.0029^{B}	0.0955 ± 0.0022^{B}	0.0321 ± 0.0028^{B}	0.1372 ± 0.0069^{B}	0.0538 ± 0.0025	0.0381 ± 0.0023^{B}
EES, 100 ng	493 ± 20	0.7272 ± 0.0177^{B}	0.2181 ± 0.0318^{B}	0.1317 ± 0.0040^{B}	0.0913 ± 0.0024^{B}	0.0360 ± 0.0023^{B}	0.1261 ± 0.0164^{B}	0.0470 ± 0.0030	0.0396 ± 0.0022^{B}

Note. BW, body weight; BS, bulbospongious; LA, levator ani; SV, seminal vesicle; HB/EP, head and body of the epididymis; T/EP, tail of the epididymis; EP fat, epididymal fat pad. Data are expressed as mean ±SEM. Means in the group ''Adolescent'' within columns with different superscripts (A–C) are significantly different ($p < 0.05$). Means in the group ADULT within columns with different superscripts (a and b) are significantly different ($p < 0.05$).

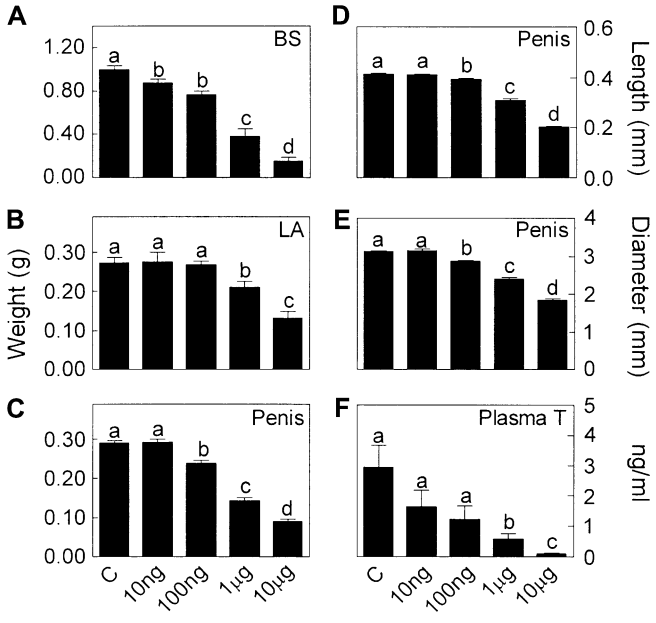

FIG. 2. The absolute weight of the bulbospongiosus muscle (A), levator ani muscle (B), and penis (C), the length of the penis (D), the diameter of the penis (E), and the concentration of plasma testosterone (F) in adult rats treated neonatally with EE at a dose of 10 ng or 100 ng or 1 µg or 10 µg/rat on every other day from postnatal days 1 to 11. Note a significant reduction in the weight of the bulbospongiosus muscle in the > 10-ng (1 µg/kg) groups, in the weight, length, and diameter of the penis in the > 100-ng groups, and in the weight of the levator ani muscle and the concentration of plasma testosterone in the > 1-µg groups. Data are expressed as mean ± SEM. Means with different superscripts are significantly ($p < 0.05$) different from controls. BS, bulbospongiosus; LA, levator ani; T, testosterone.

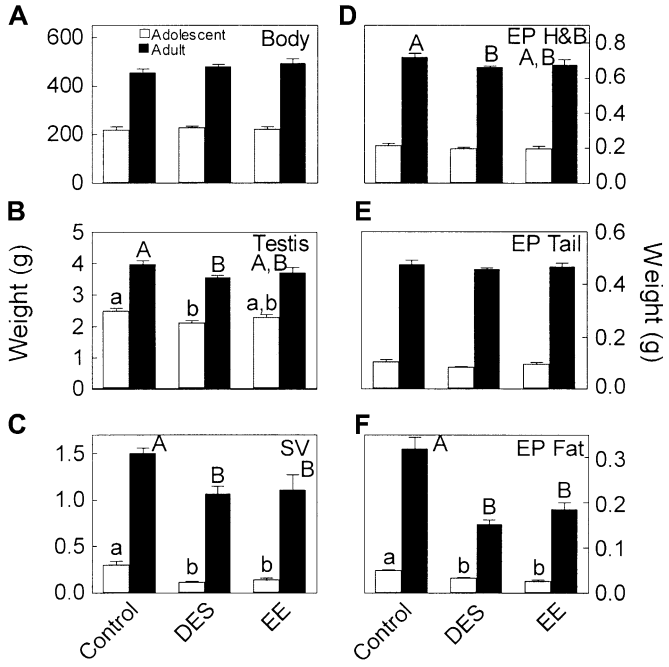

FIG. 3. The body weight (A) and the absolute paired weight of the testis (B), seminal vesicle (C), head and body of the epididymis (D), tail of the epididymis (E), and epididymal fat pad (F) in adolescent and adult rats treated neonatally with EE or DES at a dose of 100 ng/rat every day from postnatal days 1 to 6. Note that both DES and EE exposures caused a significant reduction in the weight of the seminal vesicle and epididymal fat pad in both age groups. Data are expressed as mean ± SEM. Means with different superscripts are significantly ($p < 0.05$) different from each other. SV, seminal vesicle; EP, epididymis; H&B, head and body.

both sides of these animals. In the comparative study, the epididymal fat pad weight decreased nearly 50% in both treatments and developmental stages (Fig. 3F, Table 2). The weight was not recorded in one animal each of the DES and EE groups for the same reasons described above in the dose-response study.

Penile skeletal muscles. Both absolute and relative weights of the bulbospongiosus muscle decreased significantly in a dose-dependent manner (Fig. 2A, Table 1), decreasing from 80 to 85% of the control value in the minimal dose group (10 ng) to 10–15% of the control value in the maximal dose group (10 µg). Conversely, significant weight reduction in the levator ani muscle was recorded only in the 1-µg and 10-µg groups (Fig. 2B, Table 1). Even in these groups, the level of weight reduction was much lower for the levator ani than the bulbospongiosus; for example, 25 versus 65%, respectively, in the 1-µg group, and 50 versus 90%, respectively, in the 10-µg group, compared to controls.

In the comparative study, both EE and DES treatments caused similar significant reductions in the absolute and relative weights of the bulbospongiosus muscle (Fig. 4A, Table 2), but the level of reduction for both treatments was higher at adolescence (38%) than at adulthood (25%). Conversely, the weight of the levator ani muscle did not significantly differ from controls, whether animals were treated with EE or DES or whether they were adolescent or adult (Fig. 4B, Table 2).

Penis. In addition to weight, measurements for length and diameter of the penis are also described here, for the sake of convenience. All three penile measurements, in terms of both absolute and relative values, were similar between controls and the 10-ng group (Figs. 2C–E, Table 1). However, all of them decreased significantly in a dose-dependent manner in the 100-ng, 1-µg, and 10-µg groups, although the level of decrease was comparatively higher for the weight than the length. For example, while the length decreased in the 100-ng, 1-µg, and 10-µg groups by 5, 25, and 30%, respectively, the weight decreased by 18, 50, and 70%, respectively, compared with controls.

In the comparative study, both EE and DES treatments caused similar significant reductions in penile measurements in the adolescent and adult rats, except that the penile length in the adult EE rats was not significantly different from controls (Figs. 4C–D, Table 2). Again, the level of reduction was higher for the weight than the length. For example, in the adolescent and adult rats treated neonatally with EE, while the length decreased by 17 and 4%, the weight decreased by 30 and 20%,

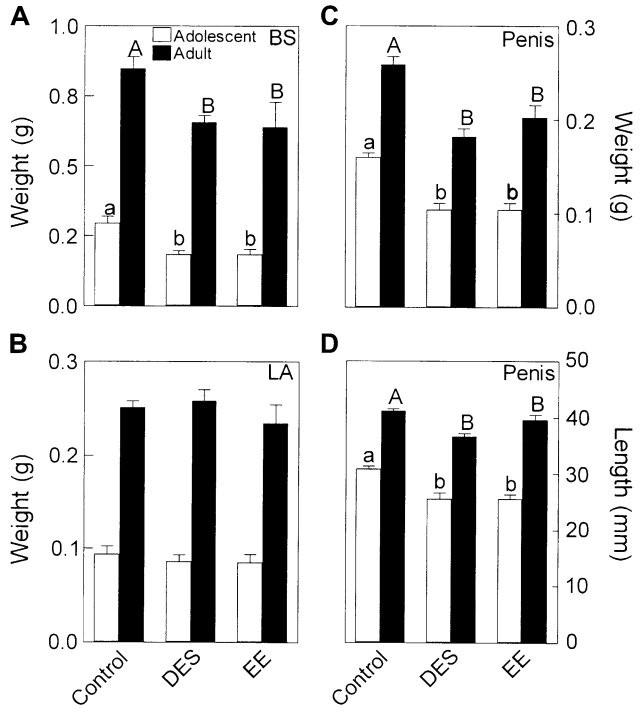

FIG. 4. The absolute weight of the bulbospongiosus (A) and levator ani (B) muscles and the absolute weight (C) and length of the penis (D) in adolescent and adult rats treated neonatally with EE or DES at a dose of 100 ng/rat every day from postnatal days 1 to 6. Note that both DES and EE exposures caused a significant reduction in the weight of the bulbospongiosus muscle and the weight and length of the penis in both age groups. Data are expressed as mean ± SEM. Means with different superscripts are significantly ($p < 0.05$) different from each other. BS, bulbospongiosus; LA, levator ani.

respectively. The corresponding figures in the adolescent and adult rats treated neonatally with DES were 17 and 11% for the length versus 35 and 30% for the weight.

Testis Descent

In the dose-response study, testes descended between 23 and 29 days of age in 5/5 rats each of the control, 10-ng and 100-ng groups, in contrast to 30–36 days of age in 4/4 rats of the 1-μg group and 51–57 days of age in 6/6 rats of the 10-μg group. In the comparative study, testes descended at 23–24 days of age in all control rats ($n = 8$), in contrast to 29–30 days of age in the 100-ng DES ($n = 8$) or EE ($n = 7$) groups.

Preputial Sheath Release

In the dose-response study, the preputial sheath was released between 44 and 50 days of age in 5/5 rats of the control group, 44–50 days of age in 3/5 rats and 51–57 days of age in 2/5 rats of the 10-ng group, and 58-64 days of age in 5/5 rats of the 100-ng group. Conversely, it was still attached at 71 days of age in 4/4 rats of the 1-μg and 6/6 rats of the 10-μg groups. In the comparative study, the preputial sheath was released at 47–49 days of age in all control rats ($n = 8$), in contrast to 65–67 days of age in three rats each in the 100-ng DES ($n = 8$) and 100-ng EE ($n = 7$) groups. It was still attached at 70 days of age, the last day of preputial sheath examination, in 5/8 rats of the DES group and 4/7 rats in the EE group.

Radiographs of the Penis

The adult penis in control rats consists of a cylindrical body, a bulbous glans, a right angle between the glans and the body, and an os penis. The latter extends from the distal end of the body to the tip of the glans and consists of a longer proximal part and a smaller distal part (Fig. 5, radiograph). Radiographs showed dose-dependent effects on the development of the os penis. Whereas both proximal and distal parts of the os penis were similarly fully developed in controls and the 10-ng group, the distal part was less developed in the 100-ng group, even lesser in the 1-μg group, and not developed at all in the 10-μg group (Fig. 5, radiograph). Similarly, compared to controls, the proximal part of the os penis was a bit thinner in the 100-ng group and increasingly thinner in the 1-μg and 10-μg groups. In addition, the penis was grossly malformed in the 1-μg and 10-μg groups, more so in the latter, characterized by rarefaction of the body and the glans and an increase in the angle between them, the glans especially acquiring a somewhat linear form.

In the comparative study, while both parts of the os penis were present in the adolescent control rats, the distal part, although less developed than that of the adult controls, was not at all developed in the adolescent DES 100-ng or EE 100-ng groups (Fig. 6, radiograph, adolescent). Likewise, at adulthood, the distal part of the os penis was less developed in both DES and EE groups as compared with controls (Fig. 6, radiograph, adult).

Histopathology and Histochemistry of the Penis

The body of the penis at puberty and adulthood in control rats consists of two corpora cavernosa that are located dorsolateral to the urethra and a corpus spongiosus that surrounds the urethra (Fig. 5, control). The corpus cavernosus consists of a network of endothelial-lined cavernous spaces (also called sinusoids), smooth muscle cells surrounding the cavernous spaces, and dense collagen fibers adjacent to muscle cells and between the cavernous spaces. Dense collagen fibers also form a part of the tunica albuginea that surrounds the corpora cavernosa. In addition, a few fat droplets are randomly seen closer to the cavernous spaces (Figs. 5 and 6, controls). The corpus spongiosus is structurally similar to the corpus cavernosus, except that it has comparatively fewer and less distended cavernous spaces, and fat droplets are absent (Fig. 7, Urethra control).

In the dose-response study, all the above described components of the corpus cavernosus were similarly developed in the control and 10-ng groups (Fig. 5, 10 ng). In the 100-ng group, there was some increase in the number of lipid droplets, but other structural components of the corpus cavernosus were not different from controls (Fig. 5, 100 ng), except in 1/5 animals, where lipid droplets predominated. Conversely, the

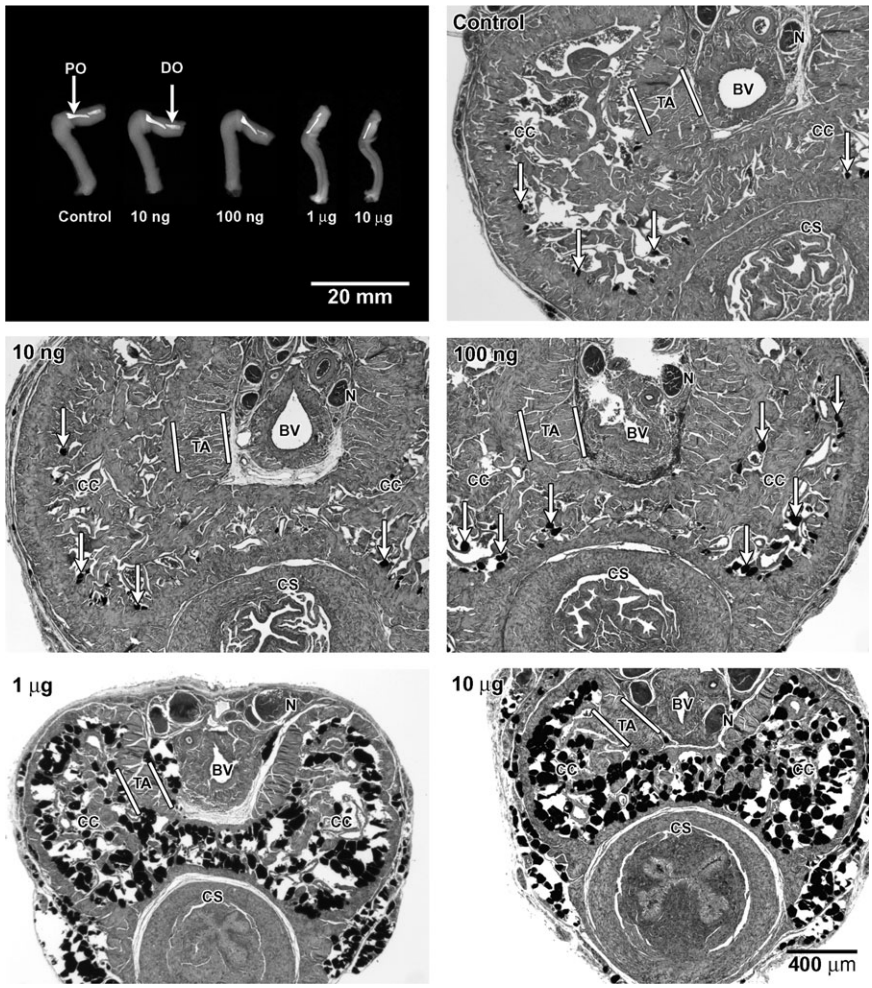

FIG. 5. Radiographs of the penis and micrographs of the body of the penis in adult rats treated neonatally with oil (control) or EE at a dose of 10 ng or 100 ng or 1 μg or 10 μg/rat on every other day from postnatal days 1 to 11. In radiographs, note grossly mal-developed penis in the 1-μg and 10-μg groups and dose-dependent underdevelopment of the proximal os penis (PO) and distal os penis (DO) in the > 100-ng groups as compared with controls and the 10-ng group. In micrographs, compared with the control and 10-ng groups, note increased number of fat cells (arrows) in the corpora cavernosa (CC) of the 100-ng group, and a nearly complete replacement of cavernous spaces and adjacent smooth muscle cells and collagen fibers by fat cells in the corpora cavernosa of the 1-μg and 10-μg groups. CS, corpus spongiosus; TA, tunica albuginea capsule; BV, blood vessel in the intercrural septum; N, nerve. En block staining with osmium tetroxide, followed by H&E staining of paraffin sections. All micrographs are of the same magnification.

corpus cavernosus in all animals of the 1-μg and 10-μg groups was replete with lipid droplets, which had replaced most of the cavernous spaces, smooth muscle cells, and collagen fibers (Fig. 5, 1 and 10 μg). Additionally, the tunica albuginea surrounding the corpora cavernosa was markedly reduced in thickness. Unlike the corpus cavernosum, the corpus spongiosus was structurally similar in the control and treated animals, regardless of the dose, except in 1/5 animals of the 10 μg in which there was an infiltration of mononuclear cells, especially lymphocytes, in the connective tissue and the urethral epithelium, which was also hypertrophied (Fig. 7, urethra EE 10 μg). In none of the treated animals, regardless of the dose, did we encounter any fat infiltration in the corpus spongiosus.

In the comparative study, the striking DES- and EE-induced effect was also infiltration by fat cells in the corpus cavernosum, although the infiltration was more pronounced at adulthood than at adolescence (Fig. 6, compare adolescent DES and EE with adult DES and EE). However, not all adult animals showed a similar level of fat infiltration. For example, while 2/5 adult animals in each of the 100-ng DES and EE groups had the level of fat infiltration similar to that observed in the 1-μg and 10-μg groups of the dose-response study, the remaining three animals had similar to that observed in the 100-ng group of the dose-response study. The corpus spongiosus was structurally similar between controls and the treated animals at both age groups, except in 1/5 animals in the EE group at adulthood in which there was lymphocytic infiltration similar to that observed in 1/5 animals of the 10-μg EE group of the dose-response study.

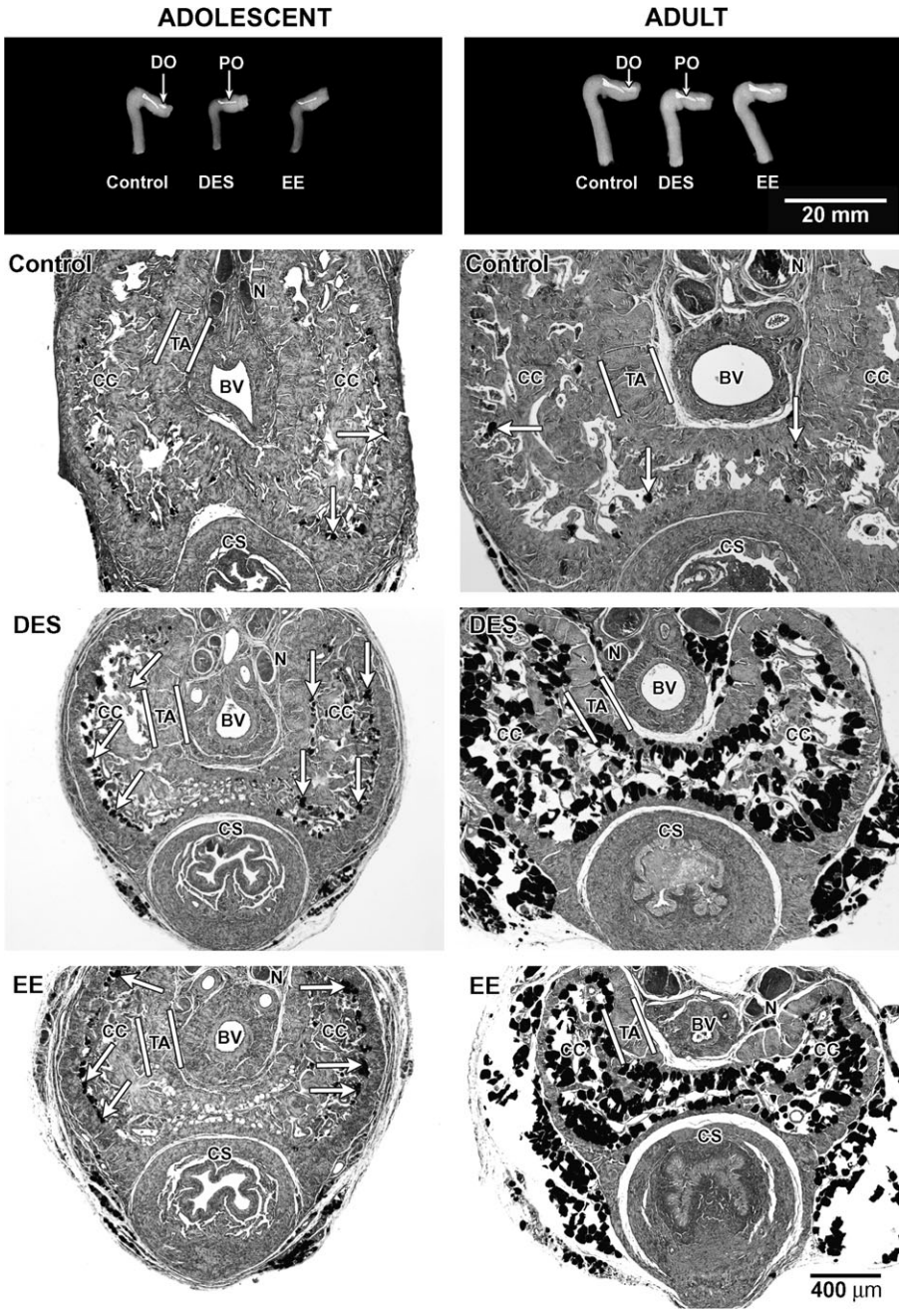

FIG. 6. Radiographs of the penis and micrographs of the body of the penis in adolescent and adult rats treated neonatally with EE or DES at a dose of 100 ng/rat every day from postnatal days 1 to 6. In radiographs, compared to controls, note that the proximal os penis (PO) is less developed and the distal os penis (DO) is not developed at adolescence in both DES and EE groups. Similarly, both groups had somewhat less developed DO at adulthood. In micrographs, note that both DES and EE treatments caused similar level of fat accumulation (arrows) in the copora cavernosa at adolescence and adulthood. CC, corpora cavernosa; CS, corpus spongiosus; TA, tunica albuginea capsule; BV, blood vessel; N, nerve. En block staining with osmium tetroxide, followed by H&E staining of paraffin sections. All micrographs are of the same magnification.

Histopathology of the Testis and Epididymis

Since the major focus of the study was the penis, histopathology of the testis and epididymis is described briefly.

Testis. For the sake of consistency and reproducibility, seminiferous tubules in stages VII and VIII of the cycle were examined to determine whether EE exposure caused pathological changes such as leukocytic infiltration, pyknosis of germ cells, sloughing of germ cells, and retention of elongated spermatids. The reason for examining stages VII and VIII was that they are androgen-dependent (Sharpe *et al.*, 1992) and contain elongated sperm lining the lumen (stage VII) or newly

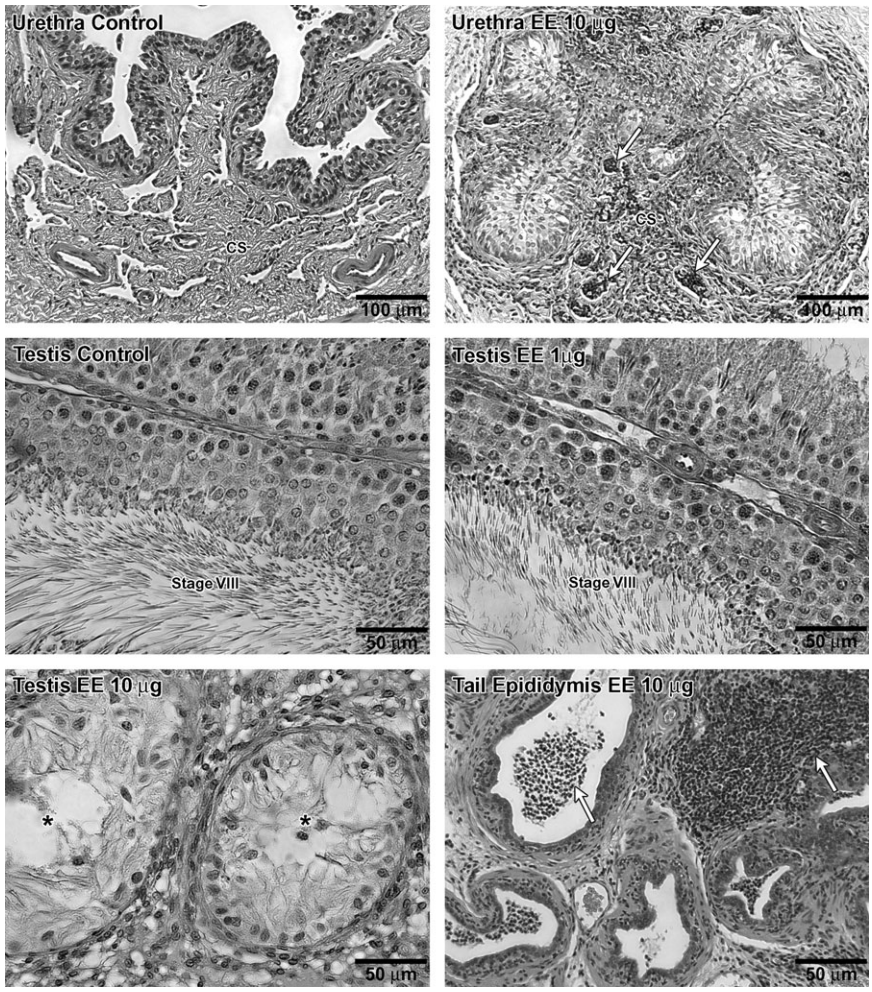

FIG. 7. Micrographs of the urethra, testis, and tail of the epididymis in adult rats treated neonatally with oil (control) or EE at a dose of 1 μg or 10 μg/rat on every other day from postnatal days 1 to 11. Urethra: Compared to controls, note leukocytic infiltration, mainly lymphocytes (arrows), in the urethral epithelium and the surrounding corpus spongiosus (CS) in 1/5 animals of the 10-μg group. Testis: Note that spermatogenesis was normal and similar in controls and all treated groups, except 2/5 animals in the 10-μg group where Sertoli cell–only seminiferous tubules (*) were prevalent. Tail of the epididymis: Note leukocytic infiltration, mainly lymphocytes (arrows), that was observed in 1/5 animals in the 1-μg group and 3/5 animals in the 10-μg group; H&E staining.

released sperm in the lumen (stage VIII). In the dose-response study, none of the treated animals in the 10-ng to 1-μg groups showed any evidence of the above-mentioned pathological changes (Fig. 7, testis control and testis EE 1 μg), except a few sloughed germ cells in the lumen, which were also seen in controls and were probably the result of immersion fixation. Conversely, in 2/5 animals of the 10-μg group, most of the seminiferous tubules contained Sertoli cells only (Fig. 7, testis EE 10 μg). As explained earlier under organ weight, the left testis in 1/4 animals in the 1-μg group and the right testis of 1 animal and both testes of another animal out of 6 animals of the 10-μg group were attached to the scrotal wall and could not be separated from the adjoining head and body of the epididymis.

In the comparative study, there was no evidence of any of the above-mentioned pathological changes, whether animals were treated with EE or DES or whether they were pubertal or adult. However, it is worth noting that both control and treated adolescent animals examined at 48–50 days of age had elongated spermatids lining the lumen of the seminiferous epithelium or free sperm in the lumen of the seminiferous tubules.

Tail of the epididymis. The right tail of the epididymis was examined only in the dose-response study for histopathology. The examination was made to determine whether there was any unusual leukocytic infiltration. None of the treated animals in the 10-ng and 100-ng groups had any evidence of increased leukocytic infiltration as compared with controls. Conversely, in 1/5 animals in the 1-μg group and 3/5 animals in the 10-μg group, the epithelium and its surrounding tissues had an intense leukocytic infiltration, mainly lymphocytes (Fig. 7, tail epididymis EE 10 μg).

Number of Sperm in the Epididymis

Sperm numbers in both the head and body and the tail of the epididymis decreased more or less in a dose-dependent manner

TABLE 3
Fertility Data of Female Rats Cohabited with Males Treated Neonatally with Various Concentrations (10 ng–10 μg) of EE on Alternate Days from Postnatal Days 1 to 11 (Dose-Response Study) and of Female Rats Cohabited with Males Treated Neonatally with EE or DES at a Dose of 100 ng Daily for Postnatal Days 1–6 (Comparative Study)

Group	Pregnant/mated	Plug positive/mated	Sperm positive/mated
Dose-response			
Control[A]	5/5	5/5	5/5
EE 10 ng[A]	5/5	5/5	5/5
EE 100 ng[A,B]	3/5	3/5	3/5
EE 1 μg[B]	0/4	0/4	0/4
EE 10 μg[B]	0/5	0/5	0/5
Comparative			
Control[A]	5/6	6/6	6/6
EE 100 ng[B]	1/6	1/6	1/6
DES 100 ng[B]	0/6	1/6	1/6

Note. Columns with different superscripts (A and B) are significantly different ($p < 0.05$).

(Fig. 1G). Compared to controls, the decrease ranged from 10 to 20% in the 10-ng group to 70–90% in the 10-μg group and was higher in the tail of the epididymis than in the head and body of the epididymis.

Plasma Testosterone

The mean concentration of plasma testosterone decreased in a dose-dependent manner from 2.96 ng/ml in controls to 1.64 ng/ml in the 10-ng group, to 1.23 ng/ml in the 100-ng group, to 0.60 ng/ml in the 1-μg group, and to 0.09 ng/ml in the 10-μg group; however, the decrease was significant only in the 1-μg and 10-μg groups (Fig. 2F).

Fertility

In the dose-dependent study, while 5/5 males each in the control and 10-ng groups deposited plugs and sperm in the vagina and sired pups, only 3/5 males did so in the 100-ng group (Table 3). Conversely, none of 4 males in the 1-μg group and none of 5 males in the 10-μg group sired pups or deposited plugs or sperm. In the comparative study, 5/6 males sired pups in the control group, in contrast to 0/6 males in the DES group and 1/6 males in the EE group. Similarly, while all control males deposited plugs and sperm, only 1 male did so in each of the DES and EE groups (Table 3).

DISCUSSION

Results of the present study showed dose-dependent effects of neonatal exposure to EE on the adult penis, with >100 ng (10 μg/kg) causing delayed preputial sheath release and maldevelopment of the penis, including malformation of the os penis, reduction in penile length, weight and diameter, and accumulation of fat cells and loss of cavernous spaces, smooth muscle cells, and collagen fibers in the corpora cavernosa of the penis. Additionally, 10 ng (1 μg/kg) and higher doses caused reductions in weight of the bulbospongiosus muscle, testis, seminal vesicle, epididymis, epididymal fat pad, and epididymal sperm numbers. The present data provide evidence that neonatal exposure to EE, as low as 10 ng (environmentally relevant dose) per day for 6 days, adversely affects a number of male reproductive organs. A ten times higher dose than this induces permanently malformed penis and infertility.

The above results are in agreement with those of a recent study in which the perinatal EE exposure from prenatal day 7 to postnatal day 18 reduced the weight of the testis and seminal vesicle at 5 μg/kg and higher doses and that of the ventral prostate, penile skeletal muscles, and glans penis at 50 μg/kg in adult Long Evans hooded rats (Howdeshell *et al.*, 2008). Conversely, Sawaki *et al.* (2003), using the same treatment protocols, did not find any adverse effects of EE in any male reproductive organs in adult CD(SD)IGS BR rats. Differences in results between the two studies could be due to differences in sensitivity of the rat strain (Howdeshell *et al.*, 2008). EE exposure at <0.2 μg/kg to pregnant CF-1 mice increased the prostate weight, increased numbers of prostatic ARs, and reduced daily sperm production (Thayer *et al.*, 2001). Similarly, EE exposure at doses lower than in contraceptive pills adversely affected reproduction in fish (Kidd *et al.*, 2007).

Other reported effects of perinatal EE exposure, albeit at higher doses, included cryptorchid testes (Walker *et al.*, 1990), gonadal dysgenesis (Yasuda *et al.*, 1985), and Leydig cell hyperplasia (Yasuda *et al.*, 1986) in mice, reduced sperm numbers, and altered sperm motion in rats (Kaneto *et al.*, 1999). Higher doses of EE (100 μg and 1 mg/kg) in the present study resulted in Sertoli cell-only seminiferous tubules and an abscessed tail of the epididymis in 2–4 out of 9 adult animals. Interestingly, none of the histopathological changes were observed at adolescence, implying their onset as a delayed phenomenon.

Data comparison between EE and DES exposures revealed that both compounds caused similar levels of reductions in weight and length of the penis, weight of the penile skeletal muscles, and weight of the testis, seminal vesicle, epididymis, and epididymal fat pad in both adolescent and adult stages. Both compounds reduced the weight of the seminal vesicle by the highest percentage and that of the head and body of the epididymis by the least percentage. Both of them delayed the development of the distal part of the os penis and induced similar levels of fat infiltration in the penis. Hence, it can be concluded that neonatal exposure to EE is as toxic to male reproductive organs as that to a known estrogenic teratogen DES. This conclusion is not surprising knowing that both DES and EE exhibited similar level of uterine growth in prepubertal

rats (Branham *et al*., 1988), binding affinity for estrogen receptors (ERs) (Fang *et al*., 2000), and prostate enlargement (Thayer *et al*., 2001).

Additional comparisons revealed important differences in sensitivity of male reproductive organs to EE exposure. Generally, seminal vesicle and epididymal fat pad were similar in sensitivity, but each one of them was more sensitive than the testis and epididymis. In our previous dose-response study, adult rats treated neonatally with DES or estradiol valerate also had maximal weight reduction in the seminal vesicle and epididymal fat pad and minimal in the testis and epididymis in all dose groups (Goyal *et al*., 2005b). Adult rats treated neonatally with estradiol benzoate had 80% reduction in the weight of seminal vesicle, in contrast to 20% in the testis (Putz *et al*., 2001). Reasons for the differential response may be attributed, in part, to differences in susceptibility among these organs to estrogens and testosterone or to levels of hormones present. These organs contain receptors for both steroids (Hess *et al*., 1997; Luke and Coffey, 1994; Pelletier *et al*., 2000), and testosterone level is decreased in all treated groups of the present study, although significantly in the 1-μg and 10-μg EE groups.

Consistent with our previous dose-response results (Goyal *et al*., 2005b), the bulbospongiosus weight was more sensitive to EE exposure than the levator ani weight, and the penile weight was more sensitive than the penile length. Actually, in terms of the level of sensitivity, bulbospogiosus muscle is at par with seminal vesicle, both of them significantly decreasing by 15% even in the lowest EE dose group (1 μg/kg) as compared with controls. Hence, the present findings, in concert with our previous findings (Goyal *et al*., 2005b), demarcate the bulbospongious weight and the penis weight as more sensitive markers than the levator ani weight and the penis length for estrogen effects. The differential response between two penile skeletal muscles may be due to differences in susceptibility to androgens because both of them are androgen dependent (Mansouri *et al*., 2003). The relatively higher percent loss in penile weight than in penile length is likely due to replacement of collagen and smooth muscle fibers by fat cells, which are obviously less dense than both fibers.

Another important finding of the study was the failure to sire pups by 40% of males in the 100-ng EE group and 100% of males in the 1-μg and 10-μg EE groups of the dose-response study and by 85–100% of males in the 100-ng EE and DES groups of the comparative study. Interestingly, all these males, except 1/6 males of the DES group, also failed to deposit copulatory plugs or sperm in the vagina, implying a compromised intromission, which likely resulted from inability of these animals to erect or maintain erection. Loss of cavernous spaces, smooth muscle cells, and collagen fibers in the corpora cavernosa, histological structures essential for erection, support this likelihood. Similar to our results, Wistar rats treated neonatally with DES at doses 1 μg and 10 μg per pup did not mate (Atanassova *et al*., 2000). A lower fertility in alligators from Lake Apopka contaminated with estrogenic pollutants is linked to an unusually small phallus (Guillette *et al*., 1996). Pubertal rabbits treated with BPA had deposition of fat and a reduction in cavernous spaces in the penis (Moon *et al*., 2001).

Mechanisms underlying estrogen-induced mal-development of the penis are not well understood. Support for an ER-mediated pathway, especially ERα pathway, comes from our own observations. ERα knockout mice exposed noenatally to DES did not develop penile abnormalities while their wild-type littermates treated similarly did (Goyal *et al*., 2007). Co-administration of ER antagonist ICI 182,780 with DES mitigated all developmental penile deformities observed in rats treated neonatally with DES alone (Goyal *et al*., 2009). Support for an AR-mediated pathway comes from our observations that coadministration of AR agonist dihydrotestosterone (DHT) or testosterone with DES prevented DES-induced developmental penile abnormalities (Goyal *et al*., 2009), as well as from another study where testosterone coadministration with DES mitigated most of the male reproductive tract abnormalities observed in rats treated neonatally with DES alone (Rivas *et al*., 2003).

Hence, it is likely that both ER and AR pathways are involved in estrogen-induced developmental deformities of the penis. One possibility is that neonatal estrogen exposure suppresses the neonatal testosterone surge, which, in turn, reprograms undifferentiated penile stromal cells toward increased adipogenesis. This possibility is supported by our findings that, while ICI coadministration with DES restores both the neonatal testosterone surge and penile deformities to within normal limits, DHT or testosterone coadministration restores only penile deformities (Goyal *et al*., 2009). Additional findings from our laboratory that ICI coadministration restores the DES-induced sixfold increase in peroxisome proliferator-activated receptor gamma (PPARγ), especially PPARγ2, a marker for differentiation of pre-adipocyte to adipocyte, to a normal level in the rat penis (Mansour *et al*., 2008) provide further support to the above possibility.

In conclusion, neonatal exposure to EE induced dose-dependent abnormalities in male reproductive organs, including penis. A dose of 10 ng (1 μg/kg), an environmentally relevant dose, caused significant reductions in the weight of the penile bulbospongiosus skeletal muscle, testis, seminal vesicle, epididymal fat pad, and epididymal sperm numbers. A ten times higher dose than this induced permanently malformed penis and infertility. Additionally, neonatal exposure to EE is as toxic to male reproductive organs, including the penis, as that to a known estrogenic teratogen DES.

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